Timing of *cyclin E* gene expression depends on the regulated association of a bipartite repressor element with a novel E2F complex

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Transient induction of the *cyclin E* gene in late G₁ gates progression into S. We show that this event is controlled via a *cyclin E* repressor module (CERM), a novel bipartite repressor element located near the *cyclin E* transcription start site. CERM consists of a variant E2F-binding site and a contiguous upstream AT-rich sequence which cooperate during G₀/G₁ to delay *cyclin E* expression until late G₁. CERM binds the protein complex CERC, which disappears upon progression through G₀–G₁ and reappears upon entry into the following G₁. CERC disappearance correlates kinetically with the liberation of the CERM module *in vivo* and *cyclin E* transcriptional induction. CERC contains E2F4/DP1 and a pocket protein, and sediments faster than classical E2F complexes in a glycerol gradient, suggesting the presence of additional components in a novel high molecular weight complex. Affinity purified CERC binds to CERM but not to canonical E2F sites, thus displaying behavior different from known E2F complexes. In cells nullizygous for members of the Rb family, CERC is still detectable and CERM-dependent repression is functional. Thus p130, p107 and pRb function interchangeably in CERC. Notably, the CERC–CERM complex dissociates prematurely in pRb^{−/−} cells in correspondence with the premature expression of *cyclin E*. Thus, we identify a new regulatory module that controls repression of G₁-specific genes in G₀/G₁.

Keywords: cell cycle/cyclin E/E2F/pocket protein/transcriptional repression

Introduction

The cyclin E/cdk2 kinase is an essential, rate-limiting regulator of the G₁ to S phase transition of the mammalian cell cycle (Keyomarsi and Herliczek, 1997). It provides a threshold for entry into the S phase, probably acting as a master switch for an unknown event essential for the initiation of DNA replication (Krude *et al.*, 1997; Hua

et al., 1997; Duronio *et al.*, 1998). *Cyclin E* expression is tightly regulated during cell cycle progression. Quiescent cells do not express cyclin E, and upon their entry into the cell cycle, the levels of *cyclin E* mRNA and protein rise sharply during a narrow window of time that precedes entry into S phase (Keyomarsi and Herliczek, 1997). Several lines of evidence suggest that this induction is controlled by the transcription factor E2F. E2F's transcriptional activity is the result of the heterodimeric association of two families of proteins, E2Fs (E2F1–6) and DPs (DP1–2) (for reviews on E2F, see Sardet *et al.*, 1997; Dyson, 1998; Nevins, 1998). They bind to DNA as free heterodimers E2F/DP or associated in larger complexes containing members of the pRB tumor suppressor family (the 'pocket proteins': pRB, p107, p130) and of the cyclin/cdk family (cyclin E/cdk2 and cyclin A/cdk2 associates physically with p107 and p130). Three successive and overlapping 'waves' of E2F complexes can be detected throughout the cell cycle, i.e. E2F/DP–pocket protein trimeric complexes that are predominant in G₀-arrested and early G₁ cells, 'free E2Fs' (E2F/DP heterodimers) with an abundance that reaches a maximum at the G₁/S phase boundary, and five component E2F complexes (E2F/DP–pocket proteins–cyclin/cdk2) which are predominant during the rest of the cell cycle and in exponentially growing cells. When bound on promoters, these E2F-complexes can act as activators ('free' E2F1-5/DP heterodimers) or as repressors (pocket protein-associated complexes). Depending on promoter context, the association of E2Fs with pocket proteins has been described to inhibit E2F/DP-mediated transactivation or, more recently, to actively repress transcription via pocket protein interactions with histone deacetylases and/or with general transcription factors.

Several lines of evidence suggest that E2F-mediated activation and repression would be involved in the control of the *cyclin E* promoter. Thus, (i) in *Drosophila*, genetic studies indicate that *cyclin E* is the limiting downstream target of an E2F-mediated transactivating event (Duronio *et al.*, 1995, 1998). (ii) In mammalian cells in culture, the disruption of E2F–pocket protein repressor complexes by viral oncoproteins adenovirus E1A (AdE1A) or herpesvirus 16 E7 (HPV16 E7) leads to a rapid induction of the endogenous *cyclin E* gene (Spitkovski *et al.*, 1994; Zerbass *et al.*, 1995). The same rapid induction is also observed following E2F1 overexpression (Degregori *et al.*, 1995; Othani *et al.*, 1995; Botz *et al.*, 1996; Geng *et al.*, 1996). (iii) The *cyclin E* gene is de-repressed and induced earlier than normal relative to S phase entry in RB^{−/−} mouse embryo fibroblasts (MEFs) (Herrera *et al.*, 1996; Hurford *et al.*, 1997) and in p107^{−/−} MEFs from a genetic background enriched for BALB/cJ (LeCouter *et al.*, 1998). (iv) The GC-rich region of the transcription initiation site of the *cyclin E* gene is highly conserved between the

mouse (Figure 2C) (Botz *et al.*, 1996) and human promoters (Othani *et al.*, 1995; Geng *et al.*, 1996) and contains several putative binding sites for SP1 and E2F. The combined mutation of two of these E2F sites in the human *cyclin E* promoter leads to partial de-repression of the promoter in G₁ (Othani *et al.*, 1995), whereas the mutation of a nearby third variant E2F site prevents the G₁/S induction of the mouse *cyclin E* promoter (Botz *et al.*, 1996).

These results are consistent with increasing evidence that some E2F-binding sites might exert a positive effect on transcription whereas others would be involved only in repression. Consistent with this, it was found that mutation of E2F binding sites within the adenovirus E2, the mammalian *DHFR* and *cyclin E* promoters block the G₁/S induction of those genes, whereas inactivation of E2F sites within the *B-myb*, *E2F1*, *E2F2*, *orc1*, *p107*, *RB1* and *cdc2* genes leads to a clear upregulation of these genes in G₀ and/or G₁ phase of the cell cycle (for review see Sardet *et al.*, 1997; Dyson, 1998; Nevins, 1998).

The molecular mechanism that determines whether these E2F sites will function as a positive or negative regulatory element is still poorly understood. In the case of the E2F-repressor sites of the *B-myb* (Lam and Watson, 1993) and *cdc2* promoters (Tommasi and Pfeifer, 1995), analyses of DNA-protein contacts by *in vivo* footprinting have shown that these sites were occupied by proteins only during G₁ when these promoters are inactive (Tommasi and Pfeifer, 1995; Zwicker *et al.*, 1996; Zwicker and Muller, 1997). Notably, they remained unoccupied after the release of repression despite the presence of transcriptionally active E2F factors in the nucleus at that time. This observation suggested that the periodic occupation of these elements by an E2F-repressor complex was likely to represent the molecular basis for the cell cycle-specific transcriptional repression of these genes (Zwicker and Muller, 1997). This also suggested that these E2F-repressor elements might display subtle characteristics which differentiate them from E2F-activator sites. Consistent with this idea, the same authors have shown that the E2F-mediated repression of the *B-myb* promoter is dependent on a contiguous co-repressor element which resembles the cell cycle homology region (CHR) (Bennett *et al.*, 1996; Liu *et al.*, 1996), a sequence previously established as being important for the cell cycle-dependent expression of the *cyclin A* and *cdc25C* genes (Lucibello *et al.*, 1995; Zwicker *et al.*, 1995). In the promoter of these genes, the CHR (5'-A/TG/TGAA-3') is cooperating with a contiguous GC-rich motif (5'-G/CGCGG-3'), termed CDE (cell cycle-dependent element) (Lucibello *et al.*, 1995; Zwicker *et al.*, 1995) or cell cycle regulatory element (CCRE) (Huet *et al.*, 1996), to form a bipartite cell cycle-regulated repressor element (Zwicker and Muller, 1997). Other similarities exist between CDE-CHR and E2F-CHR two-component repressor modules: (i) inactivation of either region of the bipartite repressor module results in high constitutive transcriptional activity of these promoters throughout the cell cycle (Zwicker and Muller, 1997); (ii) these elements are also making specific contacts with as yet unidentified proteins during G₁, when these promoters are inactive and these contacts are lost when the genes are expressed, i.e. at the G₁/S transition for *cyclin A* and *cdc2* and in late S for *cdc25C* (Lucibello *et al.*, 1995;

Tommasi and Pfeifer, 1995; Zwicker *et al.*, 1995, 1996; Huet *et al.*, 1996); (iii) finally, the CDE-CHR and E2F-CHR two-component repressor modules are both located immediately upstream of the initiation site, raising the possibility that their occupancy somehow interferes with the process of transcriptional initiation (Zwicker and Muller, 1997). Altogether, there is increasing evidence that both E2F- and CDE-mediated repression might depend on promoter-specific co-repressor elements such as CHR. However, how this co-repressor functions is still unknown.

To understand what determines the negative effect of E2F sites, we performed detailed *in vivo* and *in vitro* analyses of the transcription start site region of the mouse *cyclin E* promoter. We provide compelling evidence that one of the E2F sites located in this promoter region is part of a novel type of two-component repressor module, termed CERM (*cyclin E* repressor module). This bipartite repressor element acts as a cell cycle clock, necessary for the precise timing of *cyclin E* induction at the G₁/S transition. CERM selectively binds a novel E2F complex, termed CERC (for *cyclin E* repressor complex), the size and binding behavior of which differ from known E2F complexes. CERM/CERC dissociation correlates kinetically with *cyclin E* transcriptional induction. This finding brings new insights into the mechanism by which E2F controls the cell cycle-dependent repression of G₁-specific genes in G₀/G₁.

Results

***In vivo* occupation of a variant E2F-binding site (E2FX) and of a contiguous AT-rich sequence coincides precisely with repression of cyclin E gene transcription**

We first analyzed the cell cycle-dependent regulation of the endogenous mouse *cyclin E* promoter. Swiss 3T3 fibroblasts made quiescent by serum deprivation were stimulated to reenter the cell cycle by serum addition. Ninety-five percent of the cells synchronously proceed through a complete cell cycle over a 24 h period. The G₁ phase of the cycle takes 12 h following release from G₀ (Figures 1A and 3). Four hours later, the cells have all entered S phase, followed by G₂/M 20 h post stimulation (Figure 1A). By 24 h, the cells have passed through mitosis and re-entered G₁ (Figure 1A). *Cyclin E* mRNAs are almost undetectable in G₀ and early/mid G₁, then peak transiently at the G₁/S phase boundary and finally return to a basal level as the cells progress through G₂, M and into the G₁ phase of the next cycle (Figure 1B).

To check whether the previously identified E2F sites located in the transcription start site region of the *cyclin E* promoter were truly involved in this brief spurt of expression we performed a detailed *in vivo* analysis of the DNA-protein contacts on these sites during the cell cycle, using a dimethyl sulfate/ligation-mediated PCR (DMS/LMPCR)-based *in vivo* genomic footprinting technique (Figure 2A and B, and summary in C). Swiss 3T3 cells, were incubated with DMS at various time points following serum readdition. *In vivo* DMS-methylated DNA was then extracted and used to generate *cyclin E* promoter specific ladders by LMPCR (Mueller and Wold, 1989; Le Cam *et al.*, 1999). We designed our LMPCR primers

Fig. 1. *cyclin E* mRNA expression during synchronous cell cycle progression. (A and B) Cell cycle analysis of mouse Swiss 3T3 fibroblasts. Cells were serum-deprived for 24 h and then stimulated to re-enter the cell cycle by 10% serum addition. Cell cycle progression was checked after 0, 8, 12, 16, 20 and 24 h by: (A) FACS analysis of propidium iodide-labeled cells. Cell number (arbitrary units) was plotted against DNA content (fluorescence). (B) Expression of *cyclin E* mRNA during the cell cycle. Total RNAs prepared from Swiss 3T3 cells synchronized as above were analysed by Northern blot with 32 P-labeled cDNAs coding *cyclin E* or GAPDH.

(Figure 2C) to cover the 150 bp surrounding the major transcription start site (Botz *et al.*, 1996).

Representative genomic footprints of this region obtained with G₀ and S phase (16 h after serum stimulation) cells are shown in Figure 2A and B. In the immediate vicinity of the major initiation site, we observed two regions of DNA–protein contacts. One is a GC-rich sequence [nucleotides (nts) –30/–1] located immediately upstream of the major transcription start site, in which the G residues remain protected throughout the cell cycle (Figure 2A; termed E2FI/SP1). This constitutively protected region encompasses several overlapping SP1 binding sites and the variant E2F binding site (nts –18/–12) described previously (Botz *et al.*, 1996), termed the E2FI site. The E2FI site was shown to be important for the cell cycle activation of the mouse *cyclin E* promoter, since its mutation significantly decreased G₁/S-specific activation of the *cyclin E* promoter in synchronized NIH 3T3 cells (Botz *et al.*, 1996). The constitutive occupation of this site resembles that of other E2F DNA binding sites acting as positive regulatory elements in the DHFR (Wells *et al.*, 1996) and TK (Karlseder *et al.*, 1996) promoters.

The second region of DNA–protein contacts is located immediately downstream from the major transcription start site and is centered around another variant E2F site that we termed E2FX (nts +28/+5, TGTCCGCG). Notably, the protection of G residues within this site and the hypermethylation of a nearby guanine residue vary

during the cell cycle (Figure 2A and B). DNA–protein contacts on both DNA strands are clearly visible when the *cyclin E* promoter is silent, i.e. in serum-starved cells and during most of the G₁ phase (Figure 2A; unpublished data). In contrast, the presence of proteins on that site is barely detectable 12 and 16 h following serum addition, times which coincide precisely with the onset of cyclin E transcription in late G₁ and S (Figure 1). In addition, longer exposures of the same autoradiographs reveal a protection/occupation on a stretch of A residues (minus strand of DNA) located immediately upstream of the E2FX site (nts +20/+28) (Figure 2B). The protection of this AT-rich sequence in the minor groove of DNA parallels that of the E2FX site; it is strong in serum-starved cells and almost undetectable at 16 h following serum addition. Moreover, we also repeatedly observed that the E2FX site and the AT-rich sequence are both partially protected again 24 h post-stimulation (unpublished data), a time that coincides with the re-entry of cells into G₁ and with the complete downregulation of cyclin E transcription (Figure 1).

The DNA–protein contacts observed on this AT-E2FX region resemble those described on bipartite CDE/CCRE/E2F-CHR repressor modules in the *cyclin A*, *cdc25* and *B-myb* promoters (Lucibello *et al.*, 1995; Tommasi and Pfeifer, 1995; Zwicker *et al.*, 1995, 1996; Huet *et al.*, 1996).

Indeed, like the E2FX site, the CDE/CCRE/E2F element contacts proteins in the DNA major groove (G residues contacted) (Tommasi and Pfeifer, 1995; Zwicker *et al.*, 1995, 1996; Huet *et al.*, 1996), whereas the AT sequence and the CHR are both contacted by proteins on adenines in the minor groove (Zwicker *et al.*, 1995; Liu *et al.*, 1996). Moreover, *in vivo* occupancy of CDE/E2F and AT-E2FX sequences are both lost with kinetics, which correlates very well with the transcriptional induction of the genes (Tommasi and Pfeifer, 1995; Zwicker *et al.*, 1995, 1996; Huet *et al.*, 1996).

Thus, although the position (3' side of the E2FX site) and the sequence of the AT-rich sequence differ from that of previously described CHR elements, these results provide the first hint that the *cyclin E* promoter might be regulated by a two-component repressor module that we named CERM.

CERM is a bipartite cell cycle-regulated repressor element acting to delay cyclin E expression in late G₁

To test the role of the E2FI-site region and of the CERM, we mutated these two elements in the mouse *cyclin E* promoter. All mutant constructs, as well as the wild-type (wt) promoter, were stably transfected into Swiss 3T3 cells and their expression analyzed during the cell cycle (Figure 3). Reporter cells populations were assayed by Northern blot analysis (unpublished data) and measurements of luciferase activity (Figure 3).

The wt *cyclin E* promoter–luciferase construct (proCE) yields a 12- to 15-fold increase with kinetics that reflect the activation of the endogenous *cyclin E* gene (compare Figure 1B with 3). In contrast, the proCE– Δ E2FI/SP1 construct shows extremely low activity and is not induced in late G₁ (Figure 3). This mutation targets all the guanines protected *in vivo* within the E2FI region. We conclude that this constitutively occupied E2FI region is

Fig. 2. Identification of cell cycle-regulated protein-binding sites protected *in vivo* in the transcription start site region of the mouse *cyclin E* promoter. (A) *In vivo* footprinting of the mouse *cyclin E* promoter around the major transcription start site (black arrow) in DMS-treated Swiss 3T3 cells. Cells were synchronized as described in Figure 1. LMPCRs were performed on genomic DNA templates obtained from Swiss 3T3 cells treated with DMS. Templates were prepared at t_0 (G_0 -arrested cells) and 16 h after serum stimulation (late G_1 -S phase cells), as indicated above the lanes. The *in vitro* lane refers to similar LMPCR carried out with DMS-methylated naked DNA. Amplified DNA ladders that are visible correspond to guanosines of both strands of the mouse *cyclin E* promoter around the start site (black arrows). Protected and hyperreactive residues detected between *in vitro*- and *in vivo*-methylated DNAs are indicated as circles and stars, respectively. Putative binding sites for E2F and SP1 are indicated. (B) Although DMS reacts predominantly with G residues, it also methylates adenine (*N*-3-methyladenine) with a lower efficiency. Autoradiographs are overexposed to show the less efficient cleavage of a stretch of methylated adenine (minus strand) contiguous with the E2FX site. The protection/occupation of these bases paralleled that of the E2FX site; strong in serum-starved cells (G_0) and almost undetectable at 16 h following serum addition (S phase). (C) Summary of *in vivo* protections identified around the major transcription start site of the mouse *cyclin E* promoter. *In vivo* footprints shown above (Figure 2A and B) identify two types of DNA-protein contacts: (i) several GC-rich regions remained protected on both strand, by proteins throughout the cell cycle (labeled 'c.o.' for constitutive occupation); (ii) a region of DNA-protein contacts centered around a variant E2F site, termed E2FX, and a contiguous AT-rich sequence (nts +20/+35), together termed CERM, with a protection that varies during the cell cycle. ASCE and SCE oligonucleotides used for LMPCR are indicated.

Fig. 3. The two-component regulatory element (AT-E2FX), termed CERM, acts to delay cyclin E expression in late G₁. Swiss 3T3 fibroblasts were stably transfected with luciferase reporter constructs driven by the mouse *cyclin E* promoter (proCE) or by the corresponding CERM mutants bearing mutations within either the AT-rich sequence (proCE-ΔCERM/E2FXmut) or within the E2FX site (proCE-ΔCERM/E2FXmut). Cells were also transfected with proCE-ΔE2F1/SP1, a reporter construct driven by the *cyclin E* promoter bearing mutations within the promoter region referred to as E2F1/SP1 in Figure 2C. Neomycin-selected populations of Swiss 3T3 transfectants stably expressing these reporters were serum starved and restimulated by serum as described in Figure 1. The resulting colonies were pooled and used for luciferase assays. Luciferase activities (left axis) were plotted as the degree of stimulation relative to luciferase activity measured in quiescent cells. The results represent the average of three experiments using two independent populations of transfectants for each reporter. Histograms indicate the percentage of cells in S phase monitored in the same experiments by measurement of BrdU incorporation in DNA (right axis).

essential for the transcriptional activity of the *cyclin E* promoter. We did not pursue further the molecular analysis of this regulatory region since others have done so (Botz *et al.*, 1996; B.Vogt and P.Jansen-Durr, in preparation), but rather focused our efforts on the cell cycle-regulated element CERM.

To test the two-component repressor hypothesis, we introduced mutations separately into the two protected sequences of CERM, either the E2FX site (TGTC~~CCC~~GC→TGTA~~GAG~~C) (proCE-ΔCERM/E2Fmut) or the AT-sequence (TTTTAAAT→TCTGCAAT) (proCE-ΔCERM/ATmut). Figure 3 shows that the cell cycle kinetics of either of these promoter constructs are accelerated, i.e. they are prematurely activated in mid-G₁ compared with the wt *cyclin E* promoter. More precisely, they are still silent in quiescent cells and at early time-points following serum stimulation; however, they give rise to 12- to 15-fold increased luciferase activity 4 h earlier than the wt promoter. This functional analysis suggests that the AT-rich and the E2FX sequences both act to delay cyclin E expression in late G₁. This also suggests that together they form the bipartite cell cycle regulatory module CERM that functions solely as a transcriptional repressor element in G₀ and G₁ without affecting the level of induction at the G₁/S transition.

Identification of a cellular activity (CERC) that binds CERM in strict correlation with the silencing of cyclin E transcription

To identify the proteins that bind this novel cell cycle-regulated repressor module, we used electrophoretic mobility shift assays (EMSAs) with a DNA probe spanning this region of the mouse *cyclin E* promoter (probe CERM).

Fig. 4. EMSAs showing protein complexes (termed CERC) formed with CERM during the cell cycle. (A) A specific protein complex (referred to as CERC) binds the CERM module in quiescent Swiss 3T3 cells. Both the AT and the E2FX sequences of CERM are required to form this complex. Whole-cell extracts from serum-starved Swiss 3T3 cells were incubated with the mouse *cyclin E* DNA probe CERM (lane 1). CERC is not observed in similar EMSAs carried out with probes in which the E2FX site (CERM/mutE2FX) (lane 2) or the AT-rich sequence (CERM/mutAT) (lane 3) are mutated. (B) The binding of proteins on the CERM repressor element varies during the cell cycle. EMSAs were performed as above with whole cell extracts prepared from synchronized Swiss 3T3 cells at different time points after serum stimulation, as indicated above the lanes.

Whole-cell extracts from quiescent Swiss 3T3 cells generated a major DNA-protein complex with this probe, termed CERC (Figure 4A). The same complex was also observed using nuclear extracts (unpublished data). This complex requires both the E2FX and the AT-rich sequences, since it is not seen in EMSAs carried out with a DNA probe bearing mutations either in the E2FX site or the contiguous AT-rich sequence (Figure 4A). This is confirmed by competition using a 100-fold molar excess of wt or mutant unlabeled CERM oligonucleotides (see Figure 7B, upper right panel).

This result suggests that a cellular activity (CERC) requires both the AT-rich sequence and the E2FX site to form the complex, which is in agreement with the functional analysis of CERM (Figure 3) showing that mutating either the AT-rich or the E2FX sequences accelerates *cyclin E* promoter activation.

Since the *in vivo* occupation of CERM varied during the cell cycle, it seemed likely that CERC activity would also be cell cycle regulated. We prepared Swiss 3T3 cellular extracts at 4 h intervals during cell cycle progression and tested them by EMSAs (Figure 4B). CERC is detectable in G₀ and early/mid-G₁ phase extracts, but then disappears between late G₁ and M phase, finally reappearing as the cells progress into the next G₁ phase.

Thus, the kinetics of protein-DNA complex formation observed on CERM *in vitro* correlates perfectly with the kinetics of its *in vivo* protection and of *cyclin E* promoter

Fig. 5. CERC contains members of the E2F and pRB protein families. (A) Swiss 3T3 CERC contains p130 and p107. The following antisera were added to EMSA reactions performed as described in Figure 4: upper panel, anti-p130; central panel, anti-p107; lower panel, anti-pRB. Lane 1 in each panel indicates control EMSA carried out with G_0 cell extracts in the presence of a control antibody. Supershifted CERC is indicated as α CERC. (B and C) CERC contains the heterodimeric transcription factor E2F4/DP1. EMSAs were performed as above with extracts from quiescent Swiss 3T3 cells (lanes 1 and 2) or from cells stimulated to grow for 24 h (lanes 2 and 3) in the presence of a control antibody [(B) and (C), lanes 1 and 3], a disrupting antibody directed against E2F4 [(B), lanes 2 and 4], or a supershifting antibody against DP1 [(C), lanes 2 and 4].

silencing, thereby implicating CERC as the cell cycle-regulated repressor that mediates the CERM-dependent control of *cyclin E* gene transcription.

The repressor complex CERC contains specific members of the E2F, DP and pocket protein families

We then set out to characterize the proteins present in CERC. Although the sequence of the E2FX site (TGTCCCGC) differs from a consensus E2F-binding site sequence (TTTCGCGC), we nevertheless tested whether CERC contained E2Fs and E2F-associated proteins using antibodies directed against pocket proteins, E2Fs and DP proteins in EMSAs (Figure 5). CERC is completely supershifted by an anti-p130 antibody in G_0 and through G_1 until it disappears in S, G_2 and M phases. Interestingly, p107 almost completely replaces p130 in CERC when cells move into the next G_1 phase, 24 h after serum stimulation (Figure 5A). p107 and p130 have been shown to bind a specific subset of E2F family members, namely E2F4 and E2F5, in association with DP1 (Sardet *et al.*, 1995, 1997; Moberg *et al.*, 1996; Dyson, 1998; Nevins, 1998). Consistent with this observation, both types of CERC are almost completely disrupted by a polyclonal antibody directed against E2F4 and supershifted by an anti-DP1 (Figure 5B and C). Notably, CERC from Swiss 3T3 cells fails to react with antibodies directed against pRB (Figure 5A) or other E2F species (the presence of E2F1, E2F3 and E2F5 was also tested; unpublished data) at any phase of the cell cycle.

The pattern of EMSA complexes formed on the CERM probe is surprising, since it does not reflect the cell cycle distribution of E2F complexes usually seen with probes harboring a consensus binding site for E2F (Sardet *et al.*, 1997; Dyson, 1998; Nevins, 1998). To address this discrepancy, we also analyzed the complexes formed by our Swiss 3T3 extracts on a double-stranded oligonucleotide

Fig. 6. EMSAs showing E2F complexes formed with the E2F site of the adenovirus E2 promoter during the cell cycle. Cellular extracts prepared from synchronized Swiss 3T3 cells as described in Figure 4 were analyzed by EMSA with a double-strand oligonucleotide containing the consensus binding site for E2F from the adenovirus E2 promoter (probe: E2). Three successive 'waves' of E2F complexes (a, b and c) are observed according to their reactivity with specific antisera and their sensitivity to DOC (data not shown): (c) E2F/DP heterodimers ('free E2Fs'); (b) E2F/DP-pocket protein heterotrimers; and (a) E2F/DP-pocket protein-cyclin A/E complexes.

bearing the E2F-binding site of the adenovirus E2A promoter (Figure 6). Specificity controls, and deoxycholate (DOC) sensitivity and antibody reactivity tests were also carried out (unpublished data). The cell cycle distribution of E2F complexes in Swiss 3T3 G_0 appears to be similar to that described previously (e.g. Moberg *et al.*, 1996; Smith *et al.*, 1996).

Thus, although multiple E2F complexes can be detected throughout the cell cycle with a consensus E2F probe, the regulatory element CERM binds only a defined subset of those. Notably, it does not associate with 'free' E2Fs that are abundant in our extracts at the G_1 /S transition. This observation, and the fact that CERC always contains a member of the pocket protein family, are consistent with our mutational analyses showing that CERM exclusively mediates transcriptional repression. We conclude that the cell cycle distribution and composition of CERC is in perfect agreement with the expected behavior of the repressor bound on CERM.

CERC is a novel high molecular weight E2F complex that differs from previously described E2F complexes

What might explain the surprising binding behavior of CERM compared with 'regular' E2F sites? First, CERM might be a low affinity binding site for free E2Fs and a high affinity one for E2F-pocket protein complexes. Figure 7 shows that this is unlikely to be the case, since neither recombinant free E2Fs (E2F1/DP1, E2F4/DP1) (Sardet *et al.*, 1995) nor p130-E2F4/DP1 trimeric complex (Vairo *et al.*, 1995) are able to bind efficiently to CERM (Figure 7A, right panel), while they all bind to the E2 probe with comparably high efficiency (Figure 7A, left panel). Moreover, the E2 probe which strongly binds p130-E2F4/DP1 complexes is unable to compete for CERC binding on CERM. Indeed, Figure 7B shows that the CERM and the E2 probes poorly compete for binding to the other in gel-shift assays performed with cellular extracts prepared from G_0 -arrested Swiss 3T3 cells.

Alternatively, the cellular CERM-binding activity might also require a protein(s) in addition to E2F4/DP1 and p130 for efficient binding. Consistent with this, upon over-exposure of autoradiographs, we observed a weak complex formed between recombinant p130-E2F4/DP1 proteins

Fig. 7. CERM is a weak binding site for known E2F-complexes. (A) Recombinant E2F1/DP1, E2F4/DP1 and p130-E2F4/DP1 complexes binds CERM very weakly *in vitro*. E2F complexes were reconstituted from *in vitro* translated proteins produced in the reticulocyte lysate and analyzed by EMSAs using strictly similar amounts of the E2 probe (E2) (left panel, lanes 1–4) or of the CERM probe (right panel, lanes 5–8). Control lysate (lanes 1 and 5); E2F1/DP1 (lanes 2 and 6); E2F4/DP1 (lanes 3 and 7); p130-E2F4/DP1 (lanes 4 and 8). (B) CERM and E2 probes compete poorly for binding of cellular proteins to each other. Similar amounts of whole-cell extracts (5 µg) prepared from G₀-arrested Swiss 3T3 cells were analyzed by EMSA with a CERM probe (upper panels) or with an E2 probe (lower panels) in presence of 0-, 12-, 25-, 50- and 100-fold molar excess of unlabeled E2 or CERM competitor as indicated above the lanes. As a control, competitions were also performed with a 100-fold molar excess of E2 and CERM oligonucleotides bearing mutations within their E2F site (E2mut and CERMmut as indicated above the lanes). Cellular E2F complexes labeled as b and c are defined in Figure 6.

and the CERM probe which clearly migrated faster than cellular CERC (unpublished data).

This additional component might be part of a novel E2F complex or might bind to CERM independently. To address this point, we first tested whether the protein complex that binds CERM was larger than that of 'classical' E2F complexes. We fractionated nuclear or whole-cell extracts from G₀-arrested Swiss 3T3 cells in native glycerol gradients and tested the fractions by EMSA for both CERM (CERM probe) and whole E2F (E2 probe)-binding activities (Figure 8B and A, respectively). The bulk of E2F activity (containing mainly p130-E2F4/DP1; unpublished data; Vairo *et al.*, 1995; Moberg *et al.*, 1996;

Fig. 8. Glycerol gradient fractionation of cellular extracts shows that the CERC activity is distinct from known E2F activity. Whole-cell extracts from G₀-arrested Swiss 3T3 cells were fractionated in native 10–50% glycerol gradients. Similar amounts of indicated fractions were used for EMSAs with (A) the E2 probe or (B) with the CERM probe. E2F-complexes referred as b and c are defined in Figure 6. (C) The fractions were also analyzed by immunoblotting for the presence of p130, a protein present in both CERC and E2F complexes in G₀. (D) Distribution of molecular weight markers (BSA, thyroglobulin, apoferritin) fractionated on a similar gradient. (E) Fractionated E2F and CERC complexes formed on the E2 and CERM probes are specific for these probes. EMSA complexes formed between the E2 probe and fraction 9 (lane 1) are competed out by a 100-fold molar excess of wt unlabeled E2 probe (lane 2), but not by the corresponding mutated competitor (E2 mut) (lane 3). Similarly, CERC complex formed between CERM and fraction 11 (lane 4) is competed out by a 100-fold molar excess of wild-type CERM probe (lane 5) but not by the corresponding E2FX site-mutated competitor (CERM/E2FXmut) (lane 6).

Smith *et al.*, 1996) was detected in fractions 7–13, with a maximum in fraction 9, whereas CERC appeared in fraction 10 and persisted until fraction 16, with a maximum in fraction 11 (Figure 8B). p130 was detected by Western blot analysis in fractions 7–15, a distribution that fits both the E2F- and CERM-binding activities (Figure 8C).

Thus, the sedimentation profile of CERC in G₀ is clearly different from that of p130-E2F4/DP1 complexes that are capable of binding a 'classical' E2F site, strongly suggesting that CERC represents a distinct protein complex containing an additional protein(s). Moreover, this clear difference in size is not restricted to G₀ complexes since we also observed it in extracts of Swiss 3T3 that have quantitatively entered the G₁ phase cells (stimulated to grow for 8 h) (unpublished data).

To test further whether CERC activity corresponds to

Fig. 9. Affinity purified CERC complex is able to bind the CERM two-component regulatory element but not the consensus E2F site of the adenovirus E2 promoter. CERC was affinity purified from 1 mg of G_0 -arrested Swiss 3T3 cellular extracts using biotinylated double-stranded CERM oligonucleotides bound on streptavidin magnetic beads as described in Materials and methods. One-tenth of the CERC released from the beads was analyzed by EMSA with either the CERM probe (lane 2–4) or the E2 probe (lane 6). To show that input extracts contained CERC and known E2F-complexes, 6 μ g of G_0 -arrested Swiss 3T3 cellular extracts were also analyzed with the CERM (lane 1) or the E2 probe (lane 5). The binding of affinity purified CERC complex on CERM probe (lane 2) is competed out by a 100-fold molar excess of wt CERM probe (lane 3) but not by the corresponding E2FX site-mutated competitor (CERM/E2FXmut) (lane 4).

a novel entity that differs from known E2F complexes, we affinity purified CERC from G_0 -arrested Swiss 3T3 cellular extracts using a biotinylated CERM oligonucleotide bound on streptavidin magnetic beads. After elution from the beads, we tested the capacity of the purified complex to reassociate with a CERM and an E2 probe. The protein complexes purified reassociate with the *cyclin E* but not the E2 probe (Figure 9). We conclude that: (i) this result confirms that CERM is a low affinity binding site for 'regular' E2F complexes since they are not affinity purified by the CERM beads; (ii) CERC displays a unique specificity for CERM, i.e. a binding behavior that differs from that of known E2F complexes; and (iii) it strongly suggests that the additional proteins present in CERC are stably associated with E2F-pocket proteins and modify their DNA binding behavior, otherwise the E2F component of CERC should be able to bind the E2 probe after its release from the beads.

Altogether, these experiments show that CERC is a high molecular weight complex, stable in solution, which contains E2F4/DP1, a pocket protein and at least one additional unidentified protein. We demonstrate unambiguously that this repressor complex represents a novel E2F complex that differs in size and binding behavior from E2F complexes described previously.

The pocket proteins pRB, p107 and p130 are interchangeable in CERC

Our results suggest that, in Swiss 3T3 cells, CERC is essential for the correct timing of cyclin E expression and

contains p107 or p130 but not pRB. This conclusion is seemingly at odds with other reports showing that cyclin E expression is altered in $RB1^{-/-}$ MEFs (premature expression in G_1) but not in $p107^{-/-}$ $p130^{-/-}$ double knockout MEFs (Herrera *et al.*, 1996; Hurford *et al.*, 1997; Mulligan *et al.*, 1998). This apparent discrepancy prompted us to examine the behavior of the CERM regulatory element in these knockout cells (Figure 10).

First, we performed *in vivo* genomic footprinting and gel-shift analyses of the CERM module in serum-starved wt, $RB1^{-/-}$, $p130^{-/-}$ and $p107^{-/-}$ $p130^{-/-}$ primary MEFs (Cobrinik *et al.*, 1996; Hurford *et al.*, 1997). In each cell type, we detected *in vivo* protection of CERM (Figure 10A) and a CERC-like activity (Figure 10B). However, antibody interference assays demonstrates that the pocket protein composition of these CERCs differs. We detect predominantly p130 in wt and $RB^{-/-}$ MEFs, p107 in $p130^{-/-}$ cells and pRB in $p107^{-/-}$ $p130^{-/-}$ double knockout cells (Figure 10B). In each case, E2F4 is present in CERC. We also detect small amounts of pRB in $p107^{-/-}$ and p107 in $RB1^{-/-}$ CERC complexes. These observations are consistent with reports showing that the relative amount of the various pocket proteins varies in these knockout cells (Hurford *et al.*, 1997; Mulligan *et al.*, 1998).

The fact that a CERC-like activity containing pRB exists in $p107^{-/-}$ $p130^{-/-}$ suggests that pRB is able to compensate for the p107/p130 function in CERC-mediated repression in these cells. This provides an explanation of the normal regulation of cyclin E expression in these double knockout cells (Hurford *et al.*, 1997; Mulligan *et al.*, 1998). On the other hand, the presence of CERC on CERM in quiescent $RB^{-/-}$ cells is inconsistent with the mistiming of cyclin E expression during G_1 in this genetic background (Herrera *et al.*, 1996; Hurford *et al.*, 1997; Mulligan *et al.*, 1998).

To address this, we decided to explore further the behavior of CERM/CERC during the G_0/G_1 -S progression in pRB deficient cells.

CERC controls cyclin E expression in $RB^{-/-}$ cells but dissociates from CERM earlier than in wt cells

Luciferase reporter constructs driven by the *cyclin E* promoter (wt or mutated within CERM) were stably transfected into 3T3 cells established from MEFs deficient for pRB (M.Classon and E.Harlow, in preparation). Reporter expression was then analyzed during the cell cycle as described previously for the Swiss 3T3 transfectants. The wt *cyclin E* promoter-luciferase construct (proCE) yielded a 10-fold increase with kinetics that reflect the activation of the endogenous *cyclin E* gene in these cells, i.e. reaching a peak level of activation in G_1 several hours earlier than in wt cells (Herrera *et al.*, 1996; Hurford *et al.*, 1997) (Figure 11A). Remarkably, as in pRB positive cells [Figure 3; our unpublished results on control 3T3 fibroblasts isolated from wt MEFs (M.Classon and E.Harlow, in preparation)], these kinetics are accelerated for the CERM mutants, proCE- Δ CERM/E2FXmut and proCE- Δ CERM/ATmut (Figure 11A).

Consistent with the functional behavior of CERM in these $RB^{-/-}$ cells, this activation of the *cyclin E* promoter coincides with the dissociation of the CERC complex from CERM during the G_0/G_1 -S progression in EMSAs (Figure 11B).

Fig. 10. CERC/CERM characterization in mutant MEFs nullizygotes for one or more member(s) of the pRB family. (A) CERM is occupied *in vivo* in wt and mutant MEFs arrested in G₀. *In vivo* footprints of CERM were performed as described in Figure 2 using genomic DNAs prepared from serum starved Rb^{-/-}, p130^{-/-}, p130^{-/-} p107^{-/-} and wt MEFs treated with DMS. The DNA ladders shown correspond to the (-) strand of the +7/+49 nts region of the mouse *cyclin E* promoter. (B) Pocket proteins are interchangeable in CERC complexes in wt and mutant MEFs. EMSAs were performed as described in Figure 4 using cellular extracts prepared from wt or mutants MEFs as indicated. The presence of pocket proteins and of E2F4 in CERCs was detected as described in Figure 5 using the antibodies indicated.

Fig. 11. CERM is functional in Rb^{-/-} cells but its dissociation from CERC occurs earlier in G₁. (A) Cell cycle-dependent regulation of the wild type and CERMmut *cyclin E* promoters in Rb^{-/-} cells. 3T3 fibroblasts established from Rb^{-/-} MEFs were stably transfected with luciferase reporter constructs driven by the mouse *cyclin E* promoter (proCE) or by the corresponding CERM mutations in the AT-rich sequence (proCE-ΔCERM/E2FXmut) or the E2FX site (proCE-ΔCERM/E2FXmut) as described in Figure 3. Transfectants were serum starved and then restimulated with serum for the indicated time. Luciferase activities (left axis) were plotted as the degree of stimulation relative to luciferase activity measured in quiescent cells. Histograms indicate the percentage of cells in S phase monitored in the same experiments by measurement of BrdU incorporation in DNA (right axis). (B) EMSA showing the kinetics of CERC dissociation from CERM in Rb^{-/-} cells. EMSAs were performed as described in Figure 4 with the CERM probe and whole-cell extracts prepared from synchronized 3T3 fibroblasts established from Rb^{-/-} MEFs. Cells were synchronized as above and time points after serum stimulation are indicated above the lanes.

Altogether, these experiments demonstrate that CERM is functional in pRB deficient cells, where it acts as a dominant repressor element that controls the timing of expression of the *cyclin E* gene via its association with a CERC protein complex. Thus, the early induction of *cyclin E* in these cells compared with wt cells is clearly not due to a lack of CERM/CERC activity but rather to a premature dissociation of CERC from CERM in G₁. This finding implies that the signaling pathway that controls CERC dissociation is altered in a pRB-deficient background. Although the identity of this pRB-dependent pathway is not revealed by the present work, this provides a novel explanation for the de-regulation of cyclin E expression in Rb^{-/-} cells.

Discussion

Mammalian cyclin E is both required and rate limiting for entry into S phase of the cell cycle. Its presence is periodic, reflecting the transient activation of the *cyclin E* promoter that peaks at the G₁/S transition and then decays during S phase. Our study offers important new insights into the transcriptional mechanisms that control this cell cycle-dependent expression.

We concentrated on the role of the transcription factor E2F, which exerts positive and negative controls on G₁ cell cycle-regulated genes. It has been shown that combined mutation of two E2F sites located nearby the initiation site of transcription in the human promoter had a net positive effect on *cyclin E* promoter activity during G phase (Othani *et al.*, 1995). This observation suggested that E2F-mediated repression might control the cell cycle-dependent expression of the *cyclin E* promoter (Othani *et al.*, 1995). This model is strongly substantiated by our *in vivo* analysis of DNA-protein contacts in the mouse *cyclin E* promoter during the cell cycle. We show that only one of the E2F sites (a non-canonical E2F site referred as E2FX) located near the transcription start site is involved in repression. It functions as a dominant repressor element during G₁, acting to prevent any cyclin E expression until cells reach the G₁/S boundary. A number of criteria demonstrate that this is not simply another

example of E2F-dependent repression through a typical E2F recognition sequence (for review see Sardet *et al.*, 1997; Dyson, 1998; Nevins, 1998). The two-component architecture, the kinetics of occupation and the protein complex bound, distinguish this E2FX from canonical E2F sites.

The promoter region centered around the E2FX site was the only one which was occupied in a cell cycle-dependent fashion. The protected bases extended beyond the E2FX site to an AT-rich sequence located five nucleotides upstream. Remarkably, both sequences were bound by proteins when the *cyclin E* gene was silent (i.e. in G₀ and G₁) and no longer contacted when it was expressed (i.e. in late G₁-S). The possibility that this reflected cell cycle regulation through interaction with a repressor in G₀ and G₁ was confirmed by functional assays. That is, mutations in either the E2FX or the AT sequence led to a premature expression of the reporter gene in G₁ without changes in its level of activation. These data indicated that the AT-rich sequence and the contiguous E2FX site formed a unique regulatory element that we termed CERM, which acted solely as a transcriptional repressor. Notably, CERM has no role in the level of *cyclin E* activation at the G₁/S transition. Instead, another region of the mouse *cyclin E* promoter appeared indispensable for efficient transcription (Figure 3; Botz *et al.*, 1996). This region, located immediately upstream of the major initiation site, harbors multiple SP1 sites and possibly a variant E2F site (termed E2FI) that are constitutively occupied by proteins *in vivo* (Figure 2). Nevertheless, the repressive effect mediated by CERM is dominant over these upstream activating sequences, its main function being to delay cyclin E expression until cells reach the G₁/S boundary.

The reason why cells delay cyclin E expression is currently unknown, as is the actual functional role of the cyclin E kinase. The current model is that cyclin E regulates entry into S phase, possibly acting as a master switch for an unidentified all-or-nothing event essential for the initiation of DNA replication (Hua *et al.*, 1997; Krude *et al.*, 1997; Duronio *et al.*, 1998).

A number of characteristics displayed by CERM are similar to the class of G₀/G₁ repressor module termed CDE/E2F-CHR (Lucibello *et al.*, 1995; Zwicker *et al.*, 1995; Bennett *et al.*, 1996; Huet *et al.*, 1996; Zwicker and Muller, 1997). These include its location, pattern and kinetics of *in vivo* occupation, the cooperative function of its elements and its dominant repressive effect. However, a number of features differentiate CERM from CDE/E2F-CHR regulatory elements. First, the sequence and location of the AT-rich element in CERM clearly differs from that of the CHR. Moreover, the protein complex that interacts with CERM differs from those that have been proposed to bind on the CDE/E2F-CHR. In the B-*myb* promoter, the GC-rich motif (CDE/E2F) binds all known E2F complexes with high efficiency *in vitro* (Lam and Watson, 1993; Bennett *et al.*, 1996; Liu *et al.*, 1996; Zwicker *et al.*, 1996). However, the cell cycle pattern of *in vitro* binding (EMSA complexes) does not reflect the regulated occupation of the same CDE/E2F sequence *in vivo*, nor is it consistent with functional analyses (Bennett *et al.*, 1996). Thus, the role and nature of E2F complexes in the CDE/E2F-CHR-mediated repression of the B-*myb* promoter are still uncertain. Similarly, the role of E2Fs in the repression

of the *cdc25C*, *cdc2* and *cyclin A* genes remains highly controversial. *In vitro*, CDE/CCRE sequences either do not bind E2Fs *in vitro* (*cdc25C* gene; Liu *et al.*, 1997), or do so very weakly via E2F ternary complexes (*cdc2* and *cyclin A* gene) (Tommasi and Pfeifer, 1995; Zerfass *et al.*, 1996; Liu *et al.*, 1998). While other reports disagree with this (Zwicker *et al.*, 1995; Huet *et al.*, 1996; Philips *et al.*, 1998), these data support the notion that the whole CDE/CCRE-CHR modules in the *cdc2*, *cdc25C* and *cyclin A* promoters bind efficiently an unidentified factor, named CDF1, which is clearly distinct from E2F and does not associate directly with pocket proteins (Liu *et al.*, 1997, 1998; Zwicker *et al.*, 1997).

Our initial data also suggested that E2F might not be involved in CERM-mediated repression. Indeed, neither recombinant E2F complexes nor cellular E2F/DP heterodimers bound to a CERM probe in EMSAs while they bound strongly to a genuine E2F site (Figure 7A). Moreover, EMSA complexes formed between a CERM probe and cellular extracts were poorly competed by the E2 probe, and vice versa (Figure 7B). To our surprise, the cellular complex formed on CERM, termed CERC, unambiguously contained E2F4/DP1 and pocket proteins. Nevertheless, affinity purified CERC does not bind to typical E2F sites and it sediments as a large complex in glycerol gradients that is distinct from other E2F-containing complexes. Thus, CERC represents a novel E2F-containing multiprotein complex, the size and binding behaviors of which differ from previously described E2F complexes. Importantly, CERC fulfilled all the criteria expected of bona fide CERM-binding activity: CERC was abundant in quiescent cells and gradually disappeared upon progression through G₁ and S, with kinetics that correlates perfectly with the *in vivo* occupation of the CERM module and with the transcriptional induction of the *cyclin E* gene. Moreover, CERC reappeared as cells moved into their second G₁, extending its role in *cyclin E* regulation to exponentially growing cells. Finally, in agreement with the functional analysis of CERM, CERC is only able to bind CERM when both the AT-rich and the E2FX sequences are intact.

We propose that the CERM repressor element represents a new type of two-component repressor element which shows weak affinity for E2Fs except when E2F and pocket proteins are associated with an unidentified partner(s) within the CERC complex. The requirement for the AT-rich sequence of CERM *in vivo* and *in vitro* suggests that CERC also contains an AT-element binding protein that co-operates with E2F-pocket protein complexes to bind CERM efficiently. Possibly, its role could be to foster E2F binding by inducing local conformational changes in chromatin to allow greater E2F access. Consistent with this model, in the absence of CERC, the AT-stretch might, by virtue of its structural rigidity, assume a specific structure in DNA that decreases access to the adjacent E2FX site. This mechanism could explain why, after CERC dissociates from CERM, free E2Fs that are abundant from mid G₁ to S phase are prevented from binding to the E2FX site. Such a structural effect of AT-rich sequences has been observed in Amt1-mediated transcription (Zhu and Thiele, 1996).

CERC always contains a member of the pRB protein family. In synchronized Swiss 3T3 cells, CERC contains

exclusively p130 in quiescent cells and predominantly p107 in growing cells. On the other hand, pRB and pRB complexes are barely detectable in these cells (even using nuclear extracts) and could not be found in CERC. However, in quiescent MEFs carrying inactivated p130 and p107 alleles, CERM binds a CERC-like activity which contains pRB (Figure 10). Thus, p107, p130 and pRB are all potential components of CERC and thus are likely capable of functional compensation on CERM as they do in other functions (Dyson, 1998; Mulligan and Jacks, 1998). CERC also always contains E2F4 and DP1, in agreement with the established functional overlaps between p107, p130 and pRB in the regulation of this E2F in growing wt cells (Smith *et al.*, 1996) as well as in pocket protein deficient cells (Sardet *et al.*, 1995, 1997; Moberg *et al.*, 1996; Hurford *et al.*, 1997; Mulligan and Jacks, 1998; Mulligan *et al.*, 1998; Nevins, 1998).

The interchangeable nature of the pocket proteins in CERC is consistent with the normal cell cycle regulation of *cyclin E* expression in *p130*^{-/-} and *p107*^{-/-} *p130*^{-/-} double knockout cells (Hurford *et al.*, 1997). In direct contrast, the same cells do show de-repression of the CDE/E2F-CHR/DRS-regulated genes, *cdc2*, *E2F1*, *cyclin A* and *B-myb* (Hurford *et al.*, 1997; Mulligan *et al.*, 1998). This further supports the idea that CERM and E2F/CDE-CHR/DRS modules are regulated by distinct types of complexes.

Given the altered timing of *cyclin E* expression in *RB*^{-/-} MEFs (Figure 11A; Herrera *et al.*, 1996; Hurford *et al.*, 1997; Mulligan *et al.*, 1998), which resembles the effect of CERM mutation, we were surprised to find that CERC was intact in these cells arrested in G₀ (Figures 10 and 11). Indeed, our analysis clearly shows that the CERM/CERC repressor is still functional in these cells from G₀ and early G₁. As in wt cells, its dissociation coincides with *cyclin E* induction (Figure 11A). However, this dissociation occurs earlier in pRB-deficient cells (Figure 11B) than in other cells, strongly suggesting that the premature induction of *cyclin E* in a pRB-deficient background is a consequence of the premature dissociation of CERC from CERM in mid G₁. This finding implies that the signaling pathway that controls CERC dissociation is altered in a pRB-deficient background and provides a novel explanation for the de-regulation of *cyclin E* expression in *RB*^{-/-} cells.

In conclusion, the identification and characterization of CERM/CERC explains how some E2F binding sites can be strictly involved in transcriptional repression. Moreover, its role as a timer of *cyclin E* expression establishes this regulatory complex as a master switch which gates cell cycle progression. As such, it is possible that a number of other cell cycle genes might also be controlled by a CERC/CERM mechanism.

Materials and methods

Cell culture, cell synchronization and cell cycle analysis

Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) supplemented with 10% (v/v) fetal calf serum (FCS) (Biomed), and primary mouse fibroblasts in DMEM with 10% heat-inactivated FCS (IFCS) (Biowhitaker). 3T3 fibroblasts established from MEFs were isolated as described previously (Todaro *et al.*, 1965; M.Classon and E.Harlow, in preparation) and grown in DMEM with

10% inactivated newborn calf serum (Sigma). All these cell lines were grown at 37°C in a 5% CO₂-containing atmosphere.

For cell cycle synchronization experiments, Swiss 3T3, MEFs and 3T3 isolated from MEFs were grown to 80% confluence and made quiescent in DMEM 0% FCS for 24 h, 0.2% IFCS for 36 h, or 0% for 72 h, respectively. Cells were stimulated to re-enter the cell cycle by 10% serum addition. DNA content was analysed by FACScan (Becton Dickinson) analysis of propidium iodide-labeled cells or by indirect immunofluorescence analysis of BrdU-labeled attached cells as previously described (Sardet *et al.*, 1995; Huet *et al.*, 1996). For BrdU-labeling, synchronized cells were grown on coverslips and incubated with 100 µM bromodeoxyuridine for 1 h prior to fixation.

Isolation of the mouse cyclin E promoter and reporter plasmids, and mutagenesis

The mouse genomic library has been described previously (Cobrinik *et al.*, 1996) and was screened with the 5' end of the mouse *cyclin E* cDNA. According to the published sequence (Botz *et al.*, 1996) and proposed transcription start site, a 1 kb *Bam*HI fragment overlapping the transcription start site was cloned in the *Bg*/II site of the promoterless reporter plasmid pGL2-basic (proCE), containing the firefly luciferase gene as a reporter (Promega). *cyclin E* reporter constructs in pGL2-basic bearing the E2FX (proCE-ΔCERM/E2FXmut), AT (proCE-ΔCERM/ATmut) or E2FI-sp1 (proCE-ΔE2FI-sp1) mutations were generated by PCR with the following primers: CE/E2FX25mut, TTTTAAATGTA-GAGCTCGAAGCC; CE/TAmut, TTCTGCAATGTCCCGCTCGAA-GCCGGGCGCAGGAGCAGCC; CE/E2FI-sp1mut, GGCCGCTGAGG-GGCTCGCAGCCCTCGGTACGGTACGCGAGTACGGG-ACGGTACCG, and verified by sequencing.

Transfection, reporter assay

Swiss 3T3 cells or 3T3 fibroblasts (2×10⁵) isolated from MEFs per 6 cm-diameter dish were transfected with 0.2 µg of pCH110 (β-galactosidase expression vector), 0.2 µg of pCI-neo (neomycin resistance expression vector from Promega) and 2 µg of proCE, proCE-ΔCERM/E2FXmut, proCE-ΔCERM/mutAT or proCE-ΔE2FI-sp1 using 20 µl lipofect-AMINE (Gibco-BRL) in 2 ml of OptiMEM (Gibco-BRL) for 3 h at 37°C in a 5% CO₂ atmosphere as described by the supplier (Gibco-BRL). Neomycin-resistant populations of transfectant cells stably expressing these reporters were selected in DMEM with 10% serum and 500 µg/ml G-418 for 2 weeks. Selected cells were then serum-starved as described above and used for luciferase and β-galactosidase reporter assays at various time points following serum readdition. For luciferase assays, cell extracts prepared from 2×10⁵ cells per 6 cm-diameter dish were analyzed using standard protocols as described previously (Hateboer *et al.*, 1998). Luciferase activity values were normalized to the β-galactosidase activity to account for variations in transfection efficiencies. β-galactosidase assays were performed as described previously (Hateboer *et al.*, 1998).

Northern blot analyses

Total RNA was isolated from wt or transfected Swiss 3T3 cells by guanidinium thiocyanate-phenol/chloroform extraction. Ten micrograms of total RNA were resolved by electrophoresis on 1% agarose gel containing formaldehyde and transferred to Nylon membrane (Hybond-N; Amersham). The same membrane was successively hybridized with ³²P-labeled probes derived from human *cyclin E*, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or luciferase (firefly) full-length cDNAs.

In vivo DMS genomic footprinting

In vivo DMS genomic footprinting with the LMPCR procedure was performed essentially as described elsewhere (Hateboer *et al.*, 1998; Le Cam *et al.*, 1999). Cells (2×10⁶) per 14 cm-diameter dish were treated with the guanosine methylating agent DMS at 0.2% for 5 min at room temperature in their cell culture medium (DMEM, FCS) buffered with HEPES (20 mM final concentration), pH 7.4. After DMS treatment, cells were washed three times with cold PBS, 2% β-mercaptoethanol and then collected in 1 ml of lysis buffer (50 mM Tris pH 8, 20 mM EDTA, 1% SDS, 2% β-mercaptoethanol). Genomic DNA was isolated by three gentle extractions with phenol (pH 8) followed by two precipitations in 4 M ammonium acetate with three volumes of ethanol. DNA was then re-dissolved in 1 ml of water. As a reference, genomic DNA (1 mg/ml in water) from the same cell type was methylated *in vitro* with 0.5% DMS for 4 min at room temperature. Piperidine cleavage at methylated bases was performed in piperidine 1 N at 95°C for 30 min. Chemically cleaved samples were precipitated in ethanol, evaporated

twice and finally resuspended to 0.4 mg/ml in water. Two micrograms of cleaved genomic DNA was used for LMPCR that were carried out as described previously (Hateboer *et al.*, 1998; Le Cam *et al.*, 1999). The following primers were designed to analyze the upper and lower strands of the transcription start site region of the mouse *cyclin E* promoter: SCE4, AGCTCCTCGCTGGTCCGC; SCE5, CGGCGGCCGCTGAGGGGC; and SCE6, CGGCGGCCGCTGAGGGGCTCGC-AG-3'; ASCE4, GGATCCGCGCCTGCCCCC; ASCE5, CCCCTACACCGCGCTGGCTG; ASCE6, CCCTACACCGCGCTGGCTGTCG-AGC. All reactions were performed in presence of 10% DMSO. Temperatures for PCRs were as follows: (i) first elongation, 15 min at 76°C. (ii) PCR amplification, 4 min at 95°C followed by 30 cycles of 1 min at 95°C, 2 min at 60°C, 3 min at 76°C, ending with 7 min at 76°C; (iii) Labeling, 4 min at 95°C followed by nine cycles of 1 min at 95°C, 2 min at 60°C, 3 min at 76°C in the presence of 32 P-labeled SCE 6 or ASCE6 primer (primers were 5'-end labeled with T4 polynucleotidekinase (New England Biolabs) and [γ - 32 P]ATP (NEN)). Specific activity of the labeled primer was 3×10^6 c.p.m./pmol. Samples were loaded onto a 5% sequencing gel and run at 50 W. Dried gels were analyzed with a PhosphorImager from Molecular Dynamics, Inc.

EMSAs

Swiss 3T3 or MEF high-salt whole-cell extracts for EMSAs were prepared as follows: cells were detached with phosphate-buffered saline (PBS) and EDTA 5 mM, packed in Eppendorf tubes at 5000 g for 30 s, resuspended in two packed cell volumes of extraction buffer [20 mM HEPES pH 7.9, 0.58 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 30 mM NaF, 20 mM Na- β -glycerophosphate, 0.5 mM PMSF, 0.5 mM dithiothreitol (DTT), 16% glycerol], frozen (-70°C), then thawed (in ice) three times, and finally centrifuged at 100 000 g for 20 min (2°C). Protein concentration of supernatants was adjusted to 5 mg/ml with extraction buffer and samples were frozen at -70°C.

Recombinant E2F1, E2F4, DP1 and p130 were produced using a TNT-coupled reticulocyte lysate system (Promega) as described previously (Sardet *et al.*, 1995; Vairo *et al.*, 1995). Double-stranded probes for EMSA: E2, GCATAAGTTTCGCGCCCTTCTCAG (E2F consensus site from the adenovirus E2 promoter); E2mut, GCATAAGTTTCGATCCCTTCTCAG; CERM, CCGCGCGCTGGGATTTTAAATGTCCCGCTCGAAGCCG (nts +7/+45 of mouse *cyclin E* gene); CERM/mutE2FX, CCGCGCGCTGGGATTTTAAATGTAGAGCTCGAAGCCG; CERM/mutAT, CCGCGCGCTGGGATTCTGCAATGTCCCGCTCGAAGCCG. Probes were labeled by filling in 5' overhangs with [α - 32 P]dCTP with Klenow (Gibco-BRL) or end-labeled with T4 polynucleotide kinase (New England Biolabs) and [γ - 32 P]ATP. All probes were purified on polyacrylamide gels. EMSAs were performed as followed: 1 μ l of *in vitro*-translated proteins or 5 μ g of cell extract proteins were pre-incubated for 30 min at 22°C in 10 μ l of EMSA buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 10 mM β -glycerophosphate, 2.5 mM PNPP, 5% glycerol) and 0.5 μ g of calf thymus genomic DNA (Pharmacia). Ten femtomoles of probe at 5×10^6 c.p.m./pmol were then added for 30 min at 22°C before samples were run on 4.5% acrylamide non-denaturing gels in 0.25 $\times$ TBE at 22°C and 10 V/cm. For antibody perturbation experiments, antibodies were added to cell extracts during the pre-incubation period. Specificity of each antibody for its target was tested in preliminary experiments (data not shown), using recombinant proteins and the E2 probe. Antibodies were added as followed: anti-p130, 0.5 μ l of monoclonal RB2, (Transduction laboratories); anti-p107, 1 μ l of polyclonal C18, (Santa Cruz); anti-pRB, 1 μ l of monoclonal 21C9 (Hurford *et al.*, 1997) co-added with 0.5 μ l of polyclonal M15 (Santa Cruz); anti-E2F1, 1 μ l of polyclonal C20, (Santa Cruz); anti-E2F4, 0.1 μ l of a mouse polyclonal antiserum (Vairo *et al.*, 1995); anti-E2F3, 1 μ l of polyclonal N20, (Santa Cruz); anti-E2F5, 1 μ l of mouse monoclonal 274 (C.Sardet, unpublished reagent). Anti-DP1, 1 μ l of mouse monoclonal (E.Lam, unpublished reagent).

Native glycerol gradients

Whole-cell extracts (2.5 mg) from G₀-arrested Swiss 3T3 cells were applied to a 10–50% glycerol gradient (8 ml) in EMSA buffer without glycerol and DTT (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM NaCl). Gradients were centrifuged at 40 000 r.p.m. in an SW60 rotor for 12 h at 4°C. Samples were collected in 20 fractions of 200 μ l starting at the top of the gradient. Three microliters of each fraction was used for EMSAs with the E2 and CERM probes. Twenty microliters of each fraction were also used to performed a Western blot as follows: proteins were separated by SDS-PAGE and transferred to Immobilon P membranes (Millipore). Membranes were incubated with a monoclonal

anti-p130 antibody (RB2; Transduction Laboratories) and then subsequently incubated with a goat antimouse horseradish peroxidase-labeled antibody (Amersham). Antigen-antibody complexes were detected by enhanced chemiluminescence (ECL).

Pull down/affinity purification of CERM

CERM beads were prepared as followed: 20 pmol of 5' biotinylated double-stranded CERM oligonucleotides were incubated with 100 μ l of streptavidin magnetic beads (Dyna; suspension at 10 mg/ml) for 2 h at room temperature in binding buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1 M NaCl). CERM beads were washed three times with 1 ml of binding buffer and finally resuspended in 25 μ l of the same buffer in the presence of 100 μ g/ml bovine serum albumin (BSA). This suspension of CERM beads was then used for the pull down/affinity purification of CERM performed as follows: 2 mg of whole-cell extracts were prepared as described for EMSAs and dialyzed for 2 h at 4°C against 1 l of EMSA buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 50 mM NaCl, 1 mM DTT, 10 mM β -glycerophosphate, 2.5 mM PNPP, 5% glycerol). Half of the dialyzed extracts was then incubated with 25 μ l of CERM beads for 2 h at 4°C in the presence of 200 μ g shared calf thymus genomic DNA (Pharmacia). Beads were then washed four times with 1 ml of EMSA buffer at 4°C and finally incubated with 25 μ l of release buffer (20 mM HEPES pH 7.9, 0.45 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 30 mM NaF, 20 mM Na- β -glycerophosphate, 0.5 mM PMSF, 0.5 mM DTT, 16% glycerol) for 20 min on ice. One microliter of eluted CERM was used for EMSAs with the E2 and CERM probes. One microliter of dialyzed whole-cell extract was also used in the same EMSAs to monitor inputs of CERM and E2F complexes.

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